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BIOLOGICAL BULLETIN

FERTILIZATION AND EGG-SECRETIONS.[1]

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I.

The newer work on fertilization is directly traceable to the investigations of Frank R. Lillie.[2] It was he who first drew our attention to egg-exudates: later, the loose ends of observation and experiment united, in his mind, into a scheme whose novelty has played the part of an intellectual ferment. If we can ask new questions and for that matter, answer them too, more precisely than we could in 1913, this is largely due to the catalytic effects of the fertilizin hypothesis.

From a large list of besetting problems—most of them only solved in part—I shall select for discussion merely those that have interested me particularly, and first among these is the question whether egg-secretions have anything whatever to do with fertilization.

Skeptics, now and again, have been caught coquetting with the inevitable difficulty that egg-secretions can be discovered and analyzed in only one kind of egg at a time. By insisting on this point they appear to attach comparatively little value to the distinctions between prevalence and importance. However, we are no longer terrorized by one who brandishes the single case. Today, exudates are known not only from the eggs of one echinoderm and one annelid, but from every one of the ten species of echinoderms that have been tested. Not merely this, but additional cases have been reported from annelids, molluscs and tunicates. To the list of previously recognized instances,

[1] Read before the American Society of Zoölogists at a symposium on Fertilization, held in Chicago, December 30, 1920.

[2] An excellent summary of all the investigations in this field is to be found in Lillie's "Problems of Fertilization," University of Chicago Press, 1919.

I wish to add, parenthetically, another mollusc, the oyster, and two vertebrates, the fish, *Fundulus heteroclitus*, and the frog, *Rana pipiens*.

Though we may derive comfort from a distribution so varied, this in itself is no proof that the exudates are part and parcel of the mechanism of fertilization. Moreover the original method of demonstrating their importance is not free from suspicion. To wash eggs until all traces of their exudates have disappeared takes time; in the 18 to 36 hours necessary to accomplish this, the eggs themselves may undergo serious deterioration. Their failure therefore to develop if inseminated after washing cannot be attributed offhand to the removal of the exudates. Yet the original inference is correct[1] for the time required to remove the exudates may be cut in half by the use of running sea-water. By the use of charcoal[2] eggs may be freed from secretion completely in 3 to 4 hours; or, if the chorion is first removed, in 30 minutes. This falls well within the time limit within which unwashed eggs show no impairment of fertility. More convincing proof comes from experiments in which the fertility of eggs partially sterilized by the removal of exudate is measurably increased by the addition of freshly prepared secretion. Such an experiment with *Asterias* eggs was first carried out by Miss Woodward. Since then I have had an opportunity to verify her results on the eggs of *Echinarachnius parma*. These in a series of experiments were incompletely sterilized by the partial removal of the exudate. In one of the experiments the eggs were then divided into two lots; one of these was inseminated at once and several hours after fell into two groups—59 per cent. absolutely inactive ones and 41 per cent. in which development was going on. The latter group, however, was in turn divisible into cleavages patently anomalous and cleavages not distinguishable from the normal, approximately, in the ratio of 2 : 1. The

[1] There is one reservation; complete removal seems to have certain irreversible consequences. I have referred to the matter before. See this journal, Vol. XXVI., p. 395.

[2] The use of charcoal in this connection was suggested by Dr. G. H. A. Clowes. The suggestion was based on preliminary indications of the presence of enzymes and on the well-known efficacy of charcoal in removing these from solution. See Euler-Pope, "General Chemistry of Enzymes," p. 81.

second lot of eggs was treated with normal egg-secretion and inseminated in its presence. The results show a decrease in the inactive eggs to 50 per cent.—a decrease in the anomalous cleavages to 14 per cent. and an increase in normal divisions to 36 per cent.

II.

Our second question is, how do the exudates act? Naturally, we must formulate our explanation in terms of material entities. This, indeed, was done from the first, but the entity postulated was little more than a symbol for the observed effects.

These fall into two groups: the effects on spermatozoa and the effects on eggs. As Lillie first found, the spermatozoa are activated and exhibit a remarkable process of swarming and agglutination; the eggs, as I found, upon exposure to secretion develop spontaneously. Lillie attributed both phenomena to a single substance—the agglutinin of his earlier papers—the fertilizin of his later writings.

Lillie has introduced prevention as an aid to analysis, and has described two natural inhibitors. In the presence of one of these, the "anti-fertilizin" derived from the eggs themselves, spermatozoa do not swarm and agglutinate and eggs cannot be fertilized; in the presence of the other, contained in species-true blood, although the spermatozoa agglutinate, nevertheless the eggs fail to develop.

To account for these facts, Lillie made the following assumptions: The agglutinin has two bonds. One of these normally combines with a sperm-borne valence. The symptom of such union is the agglutination reaction. The other bond of the agglutinin unites with an egg-borne valence. The symptom of this union is the initiation of development. "Anti-fertilizin," prevents fertilization because it binds the spermophile group of the agglutinin and so renders the normal union with the sperm-valence impossible. Inasmuch as the inhibitor in the blood does not affect the agglutination reaction, this substance is assumed to occupy the ovophile bond of the agglutinin. Applying the picture-language of Ehrlich, Lillie called the agglutinin an "amboceptor," whose two bonds are satisfied in fertilization in the suggested manner.

The idea of attempting to isolate Lillie's amboceptor appeared foolhardy to many of my friends. As it was my own early attempts showed merely how it could not be done and what not to look for. The first ray of positive light came from the experiments of Richards and Miss Woodward with X-rays ('15). Their results suggested that the amboceptor might be an enzyme, and, as a matter of fact, by adapting a method previously employed by Robertson ('12) for the removal of oöcytase from ox blood, Miss Woodward succeeded in isolating a definite substance from the secretions of arbacia eggs ('18).

The substance in question was gotten in the form of a white powder easily soluble in sea water and in fresh. It did not cause the characteristic swarming and agglutination of the spermatozoa. It did however have decided virtues as a parthenogenetic agent. Moreover, it appeared to possess lipolytic properties. At any rate, oil droplets prepared from an ethereal extract of arbacia eggs, when exposed to a solution of the precipitate, decreased in diameter, in the course of two and a half hours, on the average, sixteen and seven tenths per cent. From this observation, Miss Woodward inferred that the fat was undergoing hydrolysis. Hence she called her precipitate, lipolysin.

Lipolysin is either not-agglutinin or it is denatured agglutinin. If the latter, then further search for an agglutinating substance in secretions from which lipolysin has been removed, should meet with little if any success. However a substance was isolated from just such remnants by saturation with ammonium sulfate, and the quantities so gotten appeared no smaller than those secured by the immediate saturation of the fresh exudates. The body precipitated in this fashion, when freed from salts, had no parthenogenetic properties. It did have marked powers as an agglutinant of sperm.

Whether Miss Woodward's further deductions and inferences will survive criticism matters not at all for the moment. Her results had the great virtue of preparing the ground for a different attack. In addition they made it possible to explain the effects of egg-secretions on spermatozoa and on eggs, in terms of two chemical individuals rather than in terms of one amboceptor with two side-chains.

This difference in the base from which interpretations must start, in the long run, may make small odds in the true inwardness of fertilization theory. I am not prepared to discuss the point. I am not even prepared to test the reasonableness of any particular answer pro or con for the conviction has overtaken me that I must practise birth-control in matters theoretical. But for one so minded, the one-body–two-body issue involves the whole matter of procedure. To me it is of great practical importance to determine, if possible, once for all, whether egg-secretions contain two substances or only one.

In his discussion of the question, Lillie[1] says: "This . . . does not explain why the sperm-agglutinating and the egg-activating properties of the egg-secretion always go together . . .; when the egg ceases to produce the sperm-agglutinating substance, it has lost its capacity to be activated. These two properties of the egg-secretion hang together normally; their separation under the conditions of chemical analysis may possibly denote a splitting of a single substance of the normal egg."

Unfortunately it would take us too far afield to discuss the essential question fully from the chemical side. Suffice it to say that during the last three years I have found other methods of isolation and in every case the agglutinating material and the egg-activating material are recovered as separate fractions. The amboceptor apparently has some pronounced physical weakness which causes it to break apart always at a point between the activating group and the agglutinating group. In fact this cleavage takes place so readily whenever one of the two groups combines with a precipitating agent that I have come to doubt whether the amboceptor can possibly hold together when its spermophile side-chain unites with the sperm-receptors.

However this may be, there is one bit of evidence which seems to me unassailable. Lillie proved that egg-secretions which have passed through Berkefeld filters do not cause the agglutinating reaction. The agglutinating material in this case, as Miss Sampson found last summer, and as I subsequently substantiated, can be recovered by washing the filter cone in sea-water. Whatever views one may hold regarding the physical-chemistry of

[1] "Problems of Fertilization," University of Chicago Press, p. 240.

filtration, that portion of the agglutinin which is recoverable in this manner is hardly the product of chemical breakdown suffered by the amboceptor molecule. For the ultra-conservative I may add that owing to the construction of the filter, a remnant of the secretion always fails to pass through. This remnant shows a higher agglutinating power than the original secretion. Moreover lest someone be led to suspect confusion, lipolysin can be isolated from the filtrates of such mechanical separations. Its presence in these is incidentally also revealed by activating effects on spermatozoa, a property the lipolysin shares with many other substances including preparations of pancreatic lipase.

III.

With our minds cleared on the one-body–two-body issue, we can ask and attempt to answer other specific questions. Among these we must include some further inquiries concerning the lipolysin. Is this substance really a lipolytic ferment? The evidence submitted so far is not conclusive because a decrease in the volume of oil drops might be the expression of changes in surface tension. But even if such shrinkage could be shown to result exclusively from hydrolysis, the question would still

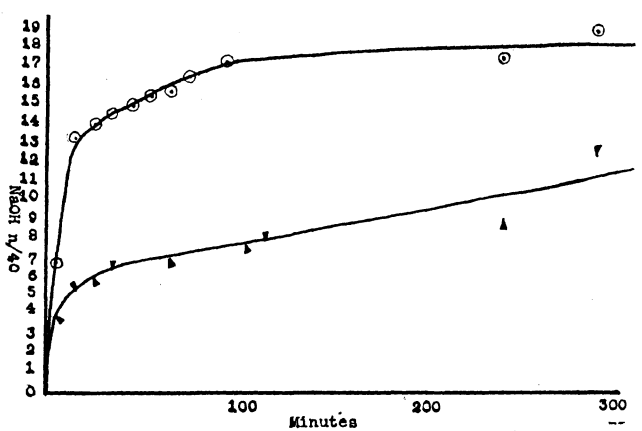

FIG. 1. Abscissa = minutes; ordinate = drops (1/100 c.c.) n/40 NaOH necessary to maintain system at PH 7. Both curves are the result of experimental averages and are slightly conventionalized. The lower one with experimental points at the apices of the triangles, represents the hydrolysis of ethyl butyrate in sea-water; the upper curve with experimental points in circles, represents the hydrolysis of ethyl butyrate in arbacia egg-secretion.

remain unsettled. An enzymatic effect is not demonstrated by hydrolysis, but by acceleration in the rate of hydrolysis.

Here again I cannot describe in detail the experiments which I have carried out during the last two years. However I am able to state very positively that the hydrolysis of neutral fats such as olive oil and whale oil as well as the cleavage of lower esters, such as ethyl butyrate are in fact accelerated not only by isolated lipolysin, but also by freshly prepared egg-secretions. The evidence, based on arbacia secretion, is given in the curves of Fig. 1. According to this lipolysin is correctly named for it is in fact a lipolytic ferment.

IV.

Can we come to similarly close quarters with the agglutinin? So far we have referred chiefly to the observable agglutination reaction. This, as Lillie found, is accompanied by other visible details. The agglutination of the spermatozoa is head-on. In *Nereis*, where the spermatozoa are larger than among the echinoderms, Lillie has described an actual swelling of the sperm-heads associated with a loss of normal refrangibility. He has suggested an increase of stickiness as the mechanism of agglutination.

The adhesiveness of cells unquestionably depends on surface properties and the effects of egg-exudates in general appear to be favorable to the view that agglutination is related to an incomplete superficial cytolysis of some sort. Aside from Lillie's evidence, which is susceptible to this interpretation, I have described a temporary agglutination of *Arenicola* larvæ accompanied by an outflow of pigment. Furthermore, isolated agglutinin has a marked effect on the surface of the egg. This quickly develops a clear zone immediately under the membrane—a result not only suggestive of membrane elevation but at the proper stage, quantitatively, perhaps the essence of this much debated process.

Richards and Miss Woodward, in their experiments with radiated egg-secretion, found evidence which suggests that very possibly the agglutinin also, is an enzyme. At any rate, radiation has opposite effects. If the secretion is radiated for two

minutes, its efficiency as a sperm-agglutinant is increased, but it is decreased if the radiation is extended to fifteen minutes. Moreover, regardless of radiation, the efficiency of the agglutinin, like that of pepsin, varies with the square root of the concentration. The presence of carbon and nitrogen may be mentioned incidentally. However, if the agglutinin is an enzyme, it is not yet possible to suggest its taxonomic position among organic catalysts. Before this can be done, we must find the process which the agglutinin catalyzes.

V.

The last question I shall discuss is the relation of lipolysin and agglutinin to the specificities of fertilization.

We know that specificity is not absolute; it is often possible to fertilize the eggs of one species with the sperm of another, yet species-true spermatozoa always fertilize a much larger percentage of eggs.

The discovery that chemical entities traceable to the eggs themselves intervene in fertilization—however obscure such intervention may appear—has exposed the problem of specificity from a new angle. Lillie has discussed the matter very thoroughly in his book.

For me, the questions involved take the following form: Is it possible to substitute the lipolysin of one species for that of another? Is it possible to do the same thing with the agglutinins? With regard to the lipolysins, the following experiments may be cited: secretion was removed from the eggs of *Echinarachnius* until their fertility had been reduced to one half. Separate lots were then inseminated in the presence of arbacia, asterias, oyster, and species-true lipolysin—all in a concentration of ten milligrams to five c.c. of sea-water.

The results are given in Table I. in which are listed, in separate columns, the percentages of inactive eggs, those of the anomalous cleavages, and those of divisions indistinguishable from the normal.. Apparently, it is not essential that the lipolysin be species-true. More dramatic than this, of course, is Miss Woodward's recently announced discovery that arbacia lipolysin is an excellent parthenogenetic agent for *Nereis* eggs.

TABLE I.

	Per Cent. Inactive Eggs.	Per Cent. Active Eggs.	
		Anomalous.	Normal.
Control	46	54	0
Arbacia lipolysin	41	57	2
Asterias lipolysin	28	66	6
Oyster lipolysin	26	62	12
Echinarachnius lipolysin	32	60	8

For the agglutinins, hybridization offers a good approach.

Just ('19) has made successful crosses between *Arbacia* and *Echinarachnius*. If now the agglutinins of these forms play a specific rôle in fertilization, one might expect that this could be rendered apparent in hybridization experiments. For this reason I treated the sex cells prior to insemination with species-true secretion and compared the results with crosses in which this treatment was not given. The experiments condensed in Table II. speak for themselves.

TABLE II.

Eggs.	Treated with Egg-Secretion.	Sperm.	Treated with Egg-Secretion.	Per Cent. Cleavage.
Arbacia	none	*Arbacia*	none	100
Arbacia	none	*Echinarachnius*	none	1
Arbacia	none	*Echinarachnius*	*Echinarachnius*	13
Arbacia	*Echinarachnius*	*Echinarachnius*	none	9
Echinarachnius	none	*Echinarachnius*	none	100
Echinarachnius	none	*Arbacia*	none	5
Echinarachnius	none	*Arbacia*	*Arbacia*	40
Echinarachnius	*Arbacia*	*Arbacia*	none	22

In these experiments I used none of the expedients so often employed to break down the incompatibilities that limit success in hybridization. Neither the eggs nor the spermatozoa were allowed to grow stale; no alkali was used; there were no repeated inseminations, nor were the spermatozoa added to the eggs in greater quantities than usual. With the sole exception of the treatment whose efficacy I wished to test, I followed strictly all the procedures of ordinary species-true insemination.

I think it must be admitted, not only that there is an effect, but that this effect applies primarily to the spermatozoa. Since

now the lipolysins are apparently non-specific, I conclude, provisionally, that the agglutinating reaction involves specific elements in the sense that species-true agglutinin may have effects quantitatively and perhaps even qualitatively different from those of the heterologous agglutinins.

Whatever conclusions we may yet reach regarding the rôle of egg-secretions, we may legitimately at this time lay claim to success in certain directions and to some extent to forecast the future. Egg-secretions are not an isolated phenomenon; they have something vital to do with the initiation of development. Finally, the period of stumbling in the utter darkness is over, for our worst methods have reached some degree of reliability and some of them have yielded definite substances whose reaction-capacities mark off the zone within which we can hope to find understanding.

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